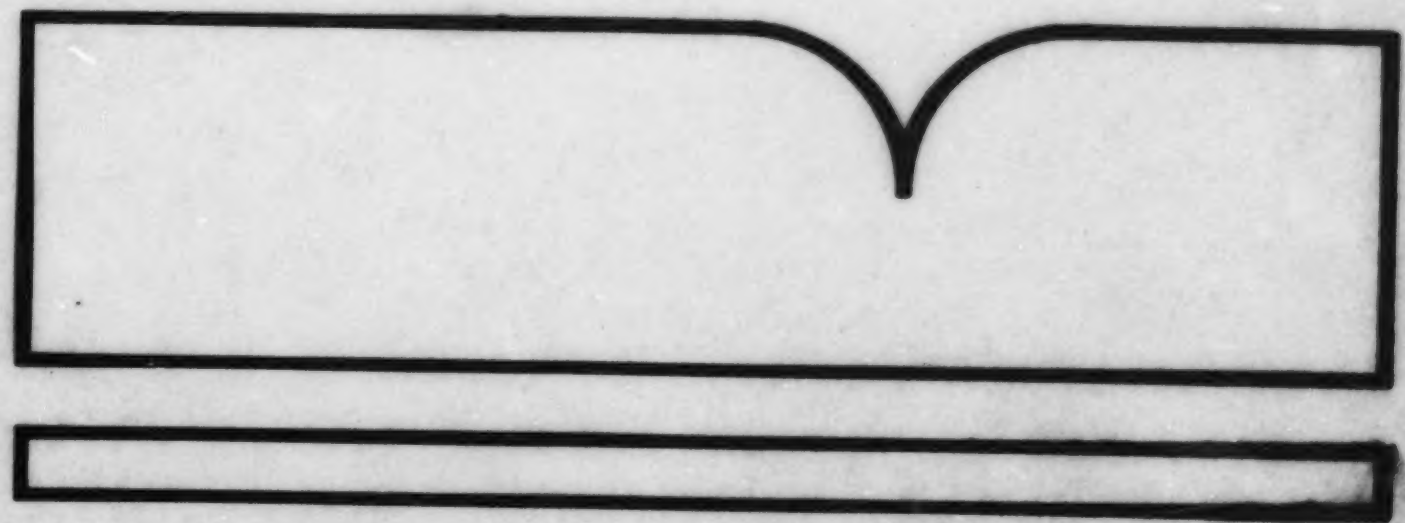
PB82-249293

Effect of Benomyl on Reproduction in the Male Rat

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

4 Nov 81



U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. REC
EPA-600/J-81-258	JOURNAL ARTICLE	PB82-249293

4. TITLE AND SUBTITLE
Effect of Benomyl on Reproduction in the Male Rat

5. REPORT DATE

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
S.D. Carter and J.W. Laskey

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Developmental Biology Division
Health Effects Research Laboratory
U.S. Envrionmental Protection Agency
Research Triangle Park, NC 27711 (U.S.A.)

10. PROGRAM ELEMENT NO.
ACSL1A

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
Office of Research and Development
Health Effects Research Laboratory
US Environmental Protection Agency
Research Triangle Park, NC 27711

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA-600/11

15. SUPPLEMENTARY NOTES
Published in: Toxicology Letters 11 (1982) 87-94

16. ABSTRACT

Adult Sprague–Dawley male rats (65 days of age) received 10 daily treatments of 0, 200 or 400 mg benomyl/kg/day by gavage. Body weight, tissue weights, total epididymal sperm counts and sperm concentration from the vas deferens were measured 14 days after the last treatment. Testicular histology was evaluated in the 0 and 400 mg/kg/day groups. Significant findings included 35–48% depressions in the total epididymal sperm counts and in the vas deferens sperm concentrations in adult animals treated with 200 or 400 mg/kg/day. Histological evaluations of testicular sections from 6 adult animals in the 400 mg/kg/day group indicated a slight to moderately severe hypospermatocytogenesis in 2 animals and a slight to severe generalized hypospermatogenesis in 2 animals. Caudal epididymis weights were significantly ($P < 0.05$) depressed with benomyl treatment. No treatment effects were found in body weight, liver, kidney, tests, or seminal vesicle weights.

less than

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to Public	Unclassified	
	20. SECURITY CLASS *(This page)* Unclassified	22. PRICE

EPA-600/J-81-258
JOURNAL ARTICLE

Toxicology Letters, 11 (1982) 87–94
Elsevier Biomedical Press

EFFECT OF BENOMYL ON REPRODUCTION IN THE MALE RAT

SUSAN D. CARTER and JOHN W. LASKEY

Developmental Biology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711 (U.S.A.)

(Received October 13th, 1981)
(Accepted November 4th, 1981)

SUMMARY

Adult Sprague–Dawley male rats (65 days of age) received 10 daily treatments of 0, 200 or 400 mg benomyl/kg/day by gavage. Body weight, tissue weights, total epididymal sperm counts and sperm concentration from the vas deferens were measured 14 days after the last treatment. Testicular histology was evaluated in the 0 and 400 mg/kg/day groups. Significant findings included 35–48% depressions in the total epididymal sperm counts and in the vas deferens sperm concentrations in adult animals treated with 200 or 400 mg/kg/day. Histological evaluations of testicular sections from 6 adult animals in the 400 mg/kg/day group indicated a slight to moderately severe hypospermatocytogenesis in 2 animals and a slight to severe generalized hypospermatogenesis in 2 animals. Caudal epididymis weights were significantly ($P < 0.05$) depressed with benomyl treatment. No treatment effects were found in body weight, liver, kidney, tests, or seminal vesicle weights.

INTRODUCTION

Benomyl*, methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate, is an important systemic fungicide registered for aerial and air blast applications and post-harvest dipping for a number of fruits, vegetables, flowers, ornamentals and shade trees [1]. As a systemic fungicide benomyl and its metabolites are actively transported from the areas of application to the growing points of the plant. Benomyl retards or prevents fungal proliferation by interfering with microtubule formation [2]. This action may inhibit such cellular processes as development and maintenance of cell shape, intracellular transport, and chromosome movements

Send reprint request to: Susan D. Carter (MD-72), Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711 (U.S.A.).

*DuPont Benlate® Benomyl Fungicide.

Abbreviations: GLM, general linear model; hCG, human chorionic gonadotropin; MBC, methyl-2-benzimidazole carbamate; PAS, periodic acid-Schiff; SAS, Statistical Analysis System.

0378-4274/82/0000–0000/$02.75 Elsevier Biomedical Press

during cell divisions [3]. Plant and mammalian microtubules have been reported to be less sensitive than those of fungi to benomyl exposure [4]. However, rapidly proliferating mammalian cells, such as those found during spermatogenesis, may still be sensitive to benomyl exposure since these cells are undergoing many of the microtubulin-dependent processes mentioned above.

The study reported here was designed to investigate the effects of benomyl exposure on the male reproduction system in the laboratory rat. Data presented includes tissue weights, measurements of total epididymal sperm and vas deferens sperm concentration, testicular histology, and hCG-stimulated serum testosterone levels from adult rats after 10 treatments of benomyl.

MATERIALS AND METHODS

65-day-old male Sprague–Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) were housed 2 per cage and allowed ad lib. access to food and tap water. Equal numbers of animals were randomly placed into groups which received 10 daily doses of either 0, 200, or 400 mg benomyl/kg/day. Animals housed together received the same benomyl exposure.

Suspensions of technical grade benomyl (E.I. duPont de Nemours & Co., Inc., Wilmington, DE) in corn oil were prepared daily. To simulate occupational exposure treatments were administered 5 times a week for a period of 2 weeks by intragastric intubation. Each animal was weighed prior to treatment and daily dosage was adjusted for body weight.

After a 14-day recovery period, all animals were weighed and injected subcutaneously with 100 IUs of hCG to stimulate the production of testosterone by the Leydig cells. 2 h after the hCG injection each animal was killed using CO_2 gas and a blood sample was collected via a cardiac puncture. In addition, the following tissues were removed and weighed: liver, kidneys, testes, seminal vesicles, adrenals, and epididymides (divided into caudate and caput sections). After weighing, each section of the epididymis was placed in 10% phosphate-buffered formalin, minced, and vortexed. Dilutions of this mixture were used for sperm counting. Samples of seminal fluid obtained from the vas deferens were weighed and immediately diluted in 10% phosphate-buffered formalin for sperm counting. Total sperm present in each sample was determined using a ZBI Coulter Counter. The accuracy of the counting system was tested using a 4-C normal hematology reference control and monosized latex particles (Coulter Electronics, Inc.). Serum samples were collected, frozen immediately ($-60\,^{\circ}C$) and forwarded to Smith Kline Clinical Laboratories, Inc. (Creve Coeur, MO) where serum testosterone levels were analyzed using a radioimmunoassay procedure [5].

The study was conducted in 2 blocks. All methods described above are applicable to block 1. Block 2 was identical to block 1 except that only 2 treatment groups (0 and 400 mg/kg/day) were used and neither adrenal weights nor serum testosterone

concentrations were determined. In addition, testicular histology was evaluated for all animals in block 2. Testes were fixed in Helly's fluid for 6–7 h, washed in running water for 20 h, and stored in 70% ethanol. Samples were then forwarded to Experimental Pathology Laboratories, Inc. (Raleigh, NC) where cross sections were prepared for histological examination using a PAS stain. Criteria used to evaluate the testicular sections were as follows: (1) focal interstitial and capsular mineralization; (2) spermatids and spermatocytes in the central lumen of the testicular tubules; (3) scattered tubules with hypospermatocytogenesis; (4) hypospermatogenesis; (5) spermatidic giant cells in the tubules; and (6) pigment in the testicular macrophages.

All data were analyzed by analysis of variance using the GLM procedure contained in SAS [6]. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Combined data from both blocks were fitted to regression models and tested using GLM. A significant ($P < 0.05$) block effect was indicated for some of the tested variables by GLM. Therefore, individual means for each block are reported in all tables presented here. Body weights, selected tissue weights and serum testosterone levels are reported in Table I. Significant ($P < 0.001$) block effects were noted in the initial body weights, liver and kidney weights. No benomyl treatment effects were found in the initial body weight or in total weight gain from the initial treatment to necropsy (26 days). In addition, no treatment effects were seen in the liver, kidney, and adrenal weights. Serum testosterone levels after stimulation with hCG were not affected by treatment.

Weights for tissues associated with the male reproduction system are presented in Table II. Overall, there were no block effects for testis, seminal vesicle, caudate, and caput weights. No treatment effects were found for seminal vesicle or caput weights. Although no significant treatment differences in mean testicular weights were seen, heterogeneity among variances for testicular weights in block 1 was found using Bartlett's Test [7]. Caudate weights were significantly ($P < 0.02$) depressed with benomyl treatment.

Table III presents treatment means for total epididymal (caudate or caput) sperm counts and vas deferens sperm concentrations. There were significant block ($P < 0.05$) and treatment effects ($P < 0.01$) in all 3 variables. Benomyl was found to cause a depression in the total number of spermatozoa present in both the caudate and caput and in sperm concentration in seminal fluid collected from the vas deferens.

Histological examinations of testes from the 0 and 400 mg/kg/day groups indicate testicular damage occurred with benomyl treatment. A slight to moderately severe hypospermatocytogenesis was found in 2 out of 6 treated animals and a slight to severe generalized hypospermatogenesis was present in the testes of 2 other

TABLE I

TEST RESULTS IN ADULT MALE SPRAGUE–DAWLEY RATS AFTER TEN ORAL TREATMENTS OF BENOMYL

Variable		Treatment (mg/kg/day)		
		0	200	400
Body weight (g) at initial treatment[c]	Block 1	292 ± 14[a] (6)[b]	290 ± 13 (5)	289 ± 17 (6)
	Block 2	335 ± 32 (4)	–	341 ± 12 (6)
Body weight gain from initial treatment to necropsy (26 d)	Block 1	106 ± 13 (6)	90 ± 38 (5)	94 ± 14 (6)
	Block 2	77 ± 12 (4)	–	88 ± 20 (6)
Liver (g)[c]	Block 1	14.4 ± 1.4 (6)	13.1 ± 1.8 (5)	13.5 ± 1.7 (6)
	Block 2	17.2 ± 2.5 (4)	–	17.9 ± 1.7 (6)
Kidneys (g)[c]	Block 1	3.47 ± .26 (6)	3.11 ± .25 (5)	3.28 ± .20 (6)
	Block 2	3.19 ± .56 (4)	–	3.29 ± .19 (6)
Adrenals (mg)	Block 1	66.5 ± 8 (6)	64.8 ± 9 (5)	62.9 ± 5 (6)
	Block 2	–	–	–
Serum testosterone (ng/dl)	Block 1	2191 ± 392 (6)	2118 ± 871 (5)	2079 ± 444 (6)
	Block 2	–	–	–

[a] Mean ± S.D.

[b] Number of samples.

[c] Significant block ($P < 0.001$) effect by GLM.

treated animals. Testicular cross sections from animals which received 0 or 400 mg/kg/day are shown in Figs. 1 and 2, respectively. A disruption of all stages of spermatogenesis appears to have occurred in the treated animal (Fig. 2). Only Sertoli cells which are usually more resistant to toxics [8] were seen around the outer parameters of the seminiferous tubules.

The sensitivity of the adult male reproductive system to benomyl is demonstrated by the sharp decrease (35–48% in the 400 mg/kg/day group) in the total number of sperm present in the caudate and caput, the depression (32–46% in the 400 mg/kg/day group) in the vas deferens sperm concentration, and by the occurrence of hypospermatogenesis in testes of animals treated with 400 mg/kg/day. Normal mammalian spermatogenesis requires interaction between hypothalamic releasing factors, pituitary gonadotrophins, and testicular androgens which control the divisions and maturation of stem cells to spermatozoa [9]. Serum testosterone in the treated animals after stimulation with hCG was not significantly different from the controls indicating that immediate disruption of the spermatogenic process is not attributed to Leydig cell destruction.

TABLE II

TISSUE WEIGHTS IN ADULT SPRAGUE–DAWLEY RATS AFTER TEN ORAL TREATMENTS OF BENOMYL

Variable		Treatment (mg/kg/day)		
		0	200	400
Testes (g)	Block 1	3.30 ± .19[a] (6)[b]	3.05 ± .42 (5)	2.99 ± .72[c] (6)
	Block 2	3.10 ± .35 (4)	–	3.41 ± .40 (6)
Seminal vesicles (g)	Block 1	1.43 ± .15 (6)	1.23 ± .09 (5)	1.33 ± .13 (6)
	Block 2	1.23 ± .15 (4)	–	1.43 ± .16 (6)
Epididymis–caput (g)	Block 1	0.629 ± .08 (6)	0.529 ± .09 (5)	0.610 ± .09 (6)
	Block 2	0.576 ± .04 (4)	–	0.528 ± .07 (6)
Epididymis–caudate (g)[d]	Block 1	0.412 ± .05 (6)	0.338 ± .06 (5)	0.362 ± .08 (6)
	Block 2	0.428 ± .03[e] (4)	–	0.329 ± .05 (6)

[a] Mean ± S.D.
[b] Number of samples.
[c] Heterogeneity of variance indicated using Bartlett's Test ($P < 0.05$).
[d] Significant treatment ($P < 0.02$) effect when data from both blocks combined by GLM.
[e] Significant treatment ($P < 0.01$) effect in each block by GLM.

TABLE III

TOTAL EPIDIDYMAL SPERM COUNTS AND VAS DEFERENS SPERM CONCENTRATION IN ADULT SPRAGUE–DAWLEY RATS AFTER TEN ORAL TREATMENTS OF BENOMYL

Variable		Treatment (mg/kg/day)		
		0	200	400
Epididymal (caput)[d,e] sperm count · 10^8/pair	Block 1	1.06 ± .23[a,c] (6)[b]	–	0.588 ± .23 (6)
	Block 2	1.32 ± .22[c] (4)	–	0.855 ± .34 (6)
Epididymal (caudate)[d,e] sperm count · 10^8/pair	Block 1	0.998 ± .16[c] (6)	0.349 ± .30 (5)	0.540 ± .36 (6)
	Block 2	1.94 ± .09[c] (4)	–	1.01 ± .62[f] (6)
Vas deferens sperm[d,e] concentration · 10^6/mg	Block 1	1.46 ± .19[c] (6)	0.899 ± .46 (5)	0.809 ± .50 (6)
	Block 2	1.91 ± .32 (4)	–	1.29 ± .65 (6)

[a] Mean ± S.D.
[b] Number of samples.
[c] Significant treatment ($P < 0.05$) effect in each block by GLM.
[d] Significant block ($P < 0.05$) effect by GLM.
[e] Significant treatment ($P < 0.01$) effect after adjustment for the block effect by GLM.
[f] Heterogeneity of variance indicated using Bartlett's Test ($P < 0.05$).

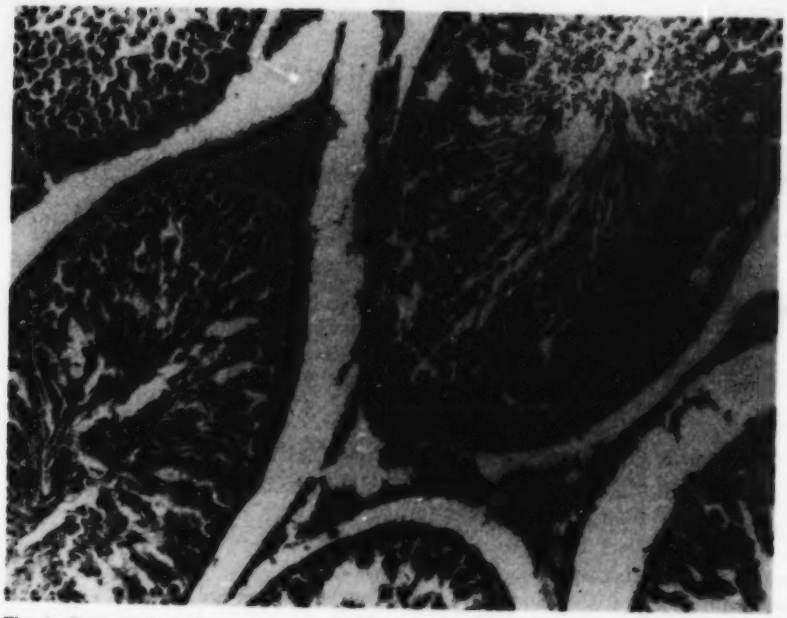


Fig. 1. Cross section of control rat testis (0 mg benomyl/kg/day for 10 days) with normal spermatogenesis occurring in all seminiferous tubules. PAS stain (× 175).

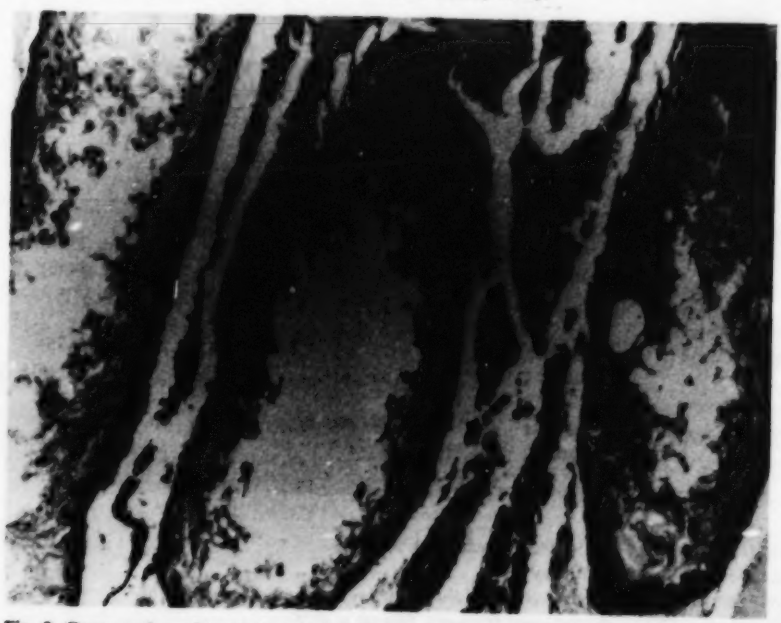


Fig. 2. Cross section of treated rat testis (400 mg benomyl/kg/day for 10 days). Note severe hypospermatogenesis with only Sertoli cells remaining near the outer parameters of the seminiferous tubules. PAS stain (× 175).

The toxicity of ingested benomyl in mammals may be attributed to one of its major metabolites, MBC [10, 11]. MBC has been reported to inhibit normal microtubulin function [2] which could disrupt many stages of spermatogenesis. For example, microtubules are a major structural component of spindle apparatus and are directly involved with chromosome movement during cellular division [3]. Therefore, a microtubule inhibitor such as MBC may interfere during the early stages of spermatogenesis when both mitotic and meiotic cell divisions occur [12]. In addition, development and maintenance of the shape of spermatozoa during the final stages of maturation (spermiogenesis) are dependent upon microtubules [3, 13].

This study was designed to evaluate potential alterations in those spermatozoa which were undergoing spermiogenesis in the seminiferous tubules of the testes during exposure. All observations were made 14 days after the last treatment. This was sufficient time for spermatozoa to travel from the tubules of the testes to the distal epididymides and vas deferens where seminal fluid was collected. During spermiogenesis, the spermatids undergo 3 developmental processes: acrosome formation, nuclear condensation, and elongation [13]. Since elongation is dependent upon microtubule formation, this process may be most sensitive to benomyl exposure. During elongation, a cylindrical array of microtubules called the manchette is formed around the caudal end of the nucleus and extends back into the region of the future neck and midpiece of the spermatozoan. The function of the manchette is not clear but it is thought to play a role in movement of cytoplasmic organelles and in mechanical protection of the spermatid during elongation [12]. Microtubules are important with respect to the structure of the tail of the spermatozoan. The core of the tail portion is composed of 2 single microtubules surrounded by 9 pairs of evenly spaced microtubules [14]. The sliding movement of this arrangement may be responsible for the motility of the spermatozoa [14]. Therefore, if benomyl acts by inhibiting microtubule formation it is likely that the reduction in sperm counts seen in this study is due to an interference during elongation. However, it is evident from the histological examinations that benomyl also interferes with the earlier stages of spermatogenesis and sperm counts would still be depressed if measured after a recovery time greater than 14 days.

Torchinskiy et al. [15] reported decreased testicular weight and absence of spermatozoa in the seminiferous tubules after chronic exposure (1000 mg/kg/day) to benomyl. In the study reported here we did not find a significant treatment effect in testicular weight when combined data from both block 1 and block 2 were analyzed. However, while no significant treatment effects were found there was a tendency for an increased sensitivity of some animals to benomyl exposure. This may be reflected in block 1 by the large variance for testicular weight in the highest benomyl treatment group (400 mg/kg/day).

In summary, subchronic exposure to benomyl did not result in any significant changes in body weight, or in liver, kidney, adrenal, testis, seminal vesicle weights, or in hCG stimulated serum testosterone levels. Caudal epididymis weights, total

sperm counts in the caudate and caput, and vas deferens sperm concentration were significantly reduced by benomyl treatment. Histological examinations revealed testicular damage in 4 out of 6 animals treated with 400 mg/kg/day. Additional studies are presently being conducted to investigate the effects on male reproduction in the Sprague–Dawley rat after exposure to benomyl during the onset of puberty.

ACKNOWLEDGEMENTS

We thank Dr. James. F. Wright and Mrs. Christine Booth for their advice and assistance with the histology and photomicrographs.

DISCLAIMER

This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

REFERENCES

1 U.S. Environmental Protection Agency Preliminary Notice – Part IX. Federal Register, Vol. 44, No. 170 (1979) 51166.
2 L.C. Davidse and W. Flach, Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*, J. Cell. Biol., 72 (1977) 174.
3 J.B. Olmsted and G.G. Borisy, Microtubules, Annu. Rev. Biochem., 42 (1973) 507.
4 C.M. Ireland, K. Gull, W.E. Gutteridge and C.I. Pogson, The interaction of benzimidazole carbamates with mammalian microtubule protein, Biochem. Pharmacol., 28 (1979) 2680.
5 J. Chen, E. Zorn, M. Hallberg and R. Wieland, Antibodies to testosterone–3-bovine serum albumin, applied to assay of serum 17B-OL androgens, Clin. Chem., 17 (1971) 581.
6 J.T. Helwig and K.A. Council (Eds.), The SAS User's Guide, SAS Institute Inc., NC, 1979.
7 J. Neter and W. Wasserman, Applied Linear Statistical Models, Richard D. Irwin, Homewood, IL, 1974, pp. 509–512.
8 W. Bloom and D.W. Fawcett, Male reproductive system, in A Textbook of Histology, 10th ed., Saunders, Philadelphia, PA, 1975, pp. 805–857.
9 W.R. Gomes, Endocrinology of the testis, in A.D. Johnson and W.R. Gomes (Eds.), The Testis, Academic Press, New York, Vol. I, 1975, pp. 2–57.
10 J.A. Gardiner, J.J. Kirland, H.L. Klopping and H. Sherman, Fate of benomyl in animals, J. Food Chem., 22 (1974) 419.
11 J.A. Styles and R. Garner, Benzimidazole carbamate methyl ester – Evaluation of its effects in vivo and in vitro, Mutation Res., 26 (1974) 177.
12 W.R. Gomes, Spermatogenesis, in A.D. Johnson and W.R. Gomes (Eds.), The Testis, Academic Press, New York, Vol. I, 1975, pp. 339–411.
13 D.W. Fawcett, W.A. Anderson and D.M. Phillips, Morphogenetic factors influencing the shape of the sperm head, Develop. Biol., 26 (1971) 220.
14 R.D. Amelar, L. Dubin and C. Schoenfeld, Sperm motility, Fertil. Steril., 34 (1980) 197.
15 A.M. Torchinskiy, N.V. Orlova, Y.B. Smirnova and L.F. Makarova, Data on the toxicological and public health characteristics of the carbamate-group pesticide benlate, Gig. Sanit., 2 (1976) 35.